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Protein-Peptide Arrays for Detection of Specific Anti-Hepatitis D Virus (HDV) Genotype 1, 6, and 8 Antibodies among HDV-Infected Patients by Surface Plasmon Resonance Imaging

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Liver diseases linked to hepatitis B-hepatitis D virus co- or superinfections are more severe than those during hepatitis B virus (HBV) monoinfection. The diagnosis of hepatitis D virus (HDV) infection therefore remains crucial in monitoring patients but is often overlooked. To integrate HDV markers into high-throughput viral hepatitis diagnostics, we studied the binding of anti-HDV antibodies (Abs) using surface plasmon resonance imaging (SPRi). We focused on the ubiquitous HDV genotype 1 (HDV1) and the more uncommon African-HDV6 and HDV8 genotypes to define an array with recombinant proteins or peptides. Full-length and truncated small hepatitis D antigen (S-HDAg) recombinant proteins of HDV genotype 1 (HDV1) and 11 HDV peptides of HDV1, 6, and 8, representing various portions of the delta antigen were grafted onto biochips, allowing SPRi measurements to be made. Sixteen to 17 serum samples from patients infected with different HDV genotypes were injected onto protein and peptide chips. In all, Abs against HDV proteins and/or peptides were detected in 16 out of 17 infected patients (94.12%), although the amplitude of the SPR signal varied. The amino-terminal part of the protein was poorly immunogenic, while epitope 65-80, exposed on the viral ribonucleoprotein, may be immunodominant, as 9 patient samples led to a specific SPR signal on peptide 65 type 1 (65#1), independently of the infecting genotype. In this pilot study, we confirmed that HDV infection screening based on the reactivity of patient Abs against carefully chosen HDV peptides and/or proteins can be included in a syndrome-based viral hepatitis diagnostic assay. The preliminary results indicated that SPRi studying direct physical HDAg–anti-HDV Ab interactions was more convenient using linear peptide epitopes than full-length S-HDAg proteins, due to the regeneration process, and may represent an innovative approach for a hepatitis syndrome–viral etiology-exploring array.

Hepatitis D virus (HDV) was discovered in 1977 as a new antigen-antibody system in liver biopsy specimens of patients chronically infected with hepatitis B virus (HBV) (1). The characterization of this infectious agent in the subsequent decade indicated that HDV is a defective (or satellite) virus of the helper virus HBV. The HDV components correspond to the core of the viral particle, whereas the envelope is entirely dependent on HBV surface antigens (HBsAg).

The characteristics of HDV distinguish it from all known animal viruses. Its small RNA genome bears resemblance only to some plant-pathogenic viroid RNAs or to cellular circular RNAs (2), and HDV is individually classified in a specific genus, *Deltavirus*. HDV is one of the smallest known human enveloped viruses. With a negative circular RNA genome varying from 1,672 to 1,697 ribonucleotides, only one protein with two isoforms bearing different carboxy-terminal ends has been regularly detected. HDV infection induces specific anti-HDAg antibodies that are detectable in the serum (3, 4). After cloning of the full-length genome, it was demonstrated that both hepatitis D antigen polypeptides, small hepatitis D antigen (S-HDAg) and large hepatitis D antigen (L-HDAg), are encoded by a single antigenomic open reading frame, due to the editing of *SHD* stop codon 196 (5, 6). During HDV genome replication, an editing mechanism catalyzed by the cellular enzyme ADAR-1 (adenosine deaminase acting on RNA) ultimately modifies the amber stop codon of the *SHD* gene to a tryptophan codon (5), leading to the extension of 19 to 20 additional codons corresponding to the C-terminal domain of the L-HDAg. Therefore, the viral genome encodes a protein with two isoforms: the small protein S-HDAg of 24 kDa contains 195 amino

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acids, and the large protein L-HDAg of 27 kDa comprises 214 amino acids. These two isoforms are associated and, together with HDV RNA, form HDV ribonucleoprotein (RNP) in infected cells and extracellular virions. The posttranslational modifications of these two isoforms include phosphorylation, acetylation, sumoylation and, for L-HDAg due to a terminal C_{211}XXQ motif, the fixation of a farnesyl group resulting from a cellular farnesyl transferase (7). It has been demonstrated that S-HDAg activates the replication of the viral genome, depending on its phosphorylation status, while L-HDAg acts as a transdominant inhibitor of replication and is involved in assembly (8, 9).

The replication of HDV is completely independent from that of HBV. Its only replication requirement is the furnishing of the viral envelope, and infection with HDV remains abortive in the absence of HBV envelope proteins. Consequently, both HBV and HDV use heparan sulfate membrane-anchored molecules and the Na taurocholate cotransporting polypeptide (NTCP) receptor to infect hepatocytes (10, 11), and the L-HBsAg protein is required for infection.

The recognition of the genetic diversity of HDV has increased in the last decade, with 8 genotypes now defined (12). HDV1 is ubiquitous, HDV2 and 4 are found in Asia, HDV3 is found in Amazonia, and HDV5 to 8, mainly of African origin, can be found elsewhere, because of migration patterns, and must therefore be taken into account (13).

Considering that the S-HDAg protein has common residues among the different genotypes and conserved secondary structures (12, 14), full-length recombinant HDV type 1 delta antigen (HDAg) is initially used to screen infected-patient total antibody responses, regardless of the infecting genotype (15). On the other hand, subdomains of the HD protein are rarely used as an antigen. In addition, the anti-peptide response might restrain immunological affinity to clade- or strain-specific linear epitopes (16). These epitopes might also be implicated in cellular adaptive immunity giving rise to different immunological responses and, by consequence, immune-mediated liver damage. Therefore, we set up a pilot study to explore the reactivities of various HDV genotype-infected samples against recombinant S-HDAg1, a highly expressed NH_2 subdomain derived from S-HDAg1 and HDAg peptide sequences derived from HDV1, HDV6, and HDV8 (Fig. 1).

MATERIALS AND METHODS

Serum samples and viral characterization. HDV-positive serum samples were selected from the collection of samples of the Associated Laboratory for the National Reference Center for hepatitis B, C, and D infection located in Bobigny, France. French law (29 November 2004) attributes to this laboratory a surveillance mission to characterize HDV isolates and to study the changes in epidemiological patterns. Serum samples collected from 17 patients age 15 to 56 years (mean, 39.7 years) and originating from Europe ($n = 2$), Asia ($n = 1$), and Africa ($n = 14$) were selected for use (see Table S1 in the supplemental material). The patients were followed in different hospitals for liver disease, and chronic HDV infection was diagnosed based on positive HBsAg detection, the presence of HDV total Ab, and the absence of anti-HBc IgM Ab. Acute HBV-HDV coinfection was not observed in this population. All serum samples were collected and kept frozen at −80°C. The samples were subjected to HDV RNA quantification by gene amplification of the *R0* region of the genome, as previously described (15, 17). No extra sampling was needed for viral genome characterization, since the HDAg sequences and HDV genotypes were determined by DNA sequencing and phylogenetic analysis, respectively.

Plasmid and recombinant *SHD* gene preparation. *SHD* sequences from HDV1 (accession no. M21012, derived from pSVLD3 [18]), HDV6 (accession no. AJ584847, from strain dFr48 [15]), and HDV8 (accession no. AM183327, derived from strain dFr2736 [13]) were amplified using Platinum *Taq* DNA polymerase high fidelity and cloned into the Champion pET Sumo TA cloning vector (Invitrogen) downstream of the His_6-Sumo fusion protein and under a strong T7 *lac* promoter. The recombinant proteins His_6-Sumo-SHD1, -6, and -8 were expressed in *Escherichia coli* strain BL21(DE3) after transformation with the respective vector, together with plasmid pRARE (Merck Biosciences) expressing tRNAs corresponding to rare codons in *E. coli*, and these were prepared after exponential growth of bacteria in Studier's autoinduction medium at 20°C overnight in the presence of kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml) (19).

A subdomain of S-HDAg1, highly expressed under the His_6 construction, was gel purified to determine the carboxy-terminal end of the domain using mass spectrometry (MS). The corresponding sequence (SHDV1 domain) was amplified and cloned by In-Fusion in the pET-28 predigested by NcoI and XhoI, and the resulting plasmid expressing the His_6-Sumo-S-HDAg1 domain was used to transform BL21(DE3).

The Sumo-SHD genes from HDV1, HDV6, and HDV8 were also subcloned by replacing the His_6 tag with a twin Strep-tag sequence (WSHPQ FEK, underlined in Fig. 1A) using a synthetic DNA replacing the XbaI-EcoRI fragment of the Champion pET Sumo TA cloning vector, thereby allowing for quick and mild purification of the corresponding fusion proteins using affinity chromatography on a Strep-Tactin resin (IBA GmbH).

Sumo-protease digestion was optimized after a dialysis step using Zeba Spin desalting columns and the 10 U of Sumo protease per mmol of His_6-Sumo-S-HDAg1.

Recombinant protein analyses. The transcription experiments were conducted *in vitro* under both bacterial and eukaryotic conditions. To evaluate recombinant protein reactivity, a commercial enzyme-linked immunosorbent assay (ELISA) was performed using the EIAgenHDV Ag kit from Adaltis using an anti-HDV monoclonal mouse Ab. Polyacrylamide gel electrophoresis (PAGE) and Western blot detections were performed using Coomassie blue staining and human anti-HDV polyclonal antibodies (a kind gift from Camille Sureau), respectively. Briefly, recombinant S-HDAg proteins were submitted to electrophoresis at 130 mV in a 12.5% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R-250. A duplicate gel was transferred to a nitrocellulose membrane using semiwet transfer (Bio-Rad) for 45 min at 400 mA in CAPS (3-[cyclohexamino]-1-propanesulfonic acid) buffer. The transfer was checked using Ponceau red. After blocking with 5% TBSTM (Tris-buffered saline [TBS], 0.3% Tween 20, 5% nonfat milk), the membrane was incubated overnight at 4°C using human anti-HDV polyclonal antibodies diluted 1:1,000 in TBS–0.3% Tween 20. After TBST washing, the membrane was incubated for 2 h at room temperature with conjugated anti-human antibody (GE Healthcare) diluted 1:5,000 in 5% TBSTM. After the washings, the membrane was revealed using enhanced chemiluminescence and exposed to a film.

Preparation of reagents for SPR analysis. Pyrrole-protein conjugates were generated as described previously (20). Peptides were synthesized by Altergen (Bischheim, France) with a pyrrole-modified NH_2 terminus, as previously described (20). Pyrrole allows the grafting of probes on the gold surface of the prism array; it polymerizes under electricity power (21). Eleven peptides were derived from HDV of various genotypes, one from hepatitis C virus (HCV), and one from hen egg lysozyme (HEL). The full sequences can be found on the ExPASy server (see http://ca.expasy.org; see also Table S2 in the supplemental material). HEL, human IgG, and human IgM were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The antibodies against human IgG and human IgM were from Valbiotech (Paris, France) and Jackson (Newmarket, United Kingdom), respectively. Rabbit immune serum against HCV peptide was prepared by NeoMPS (Strasbourg, France).

Glass prisms coated with a 50-nm gold layer were obtained from

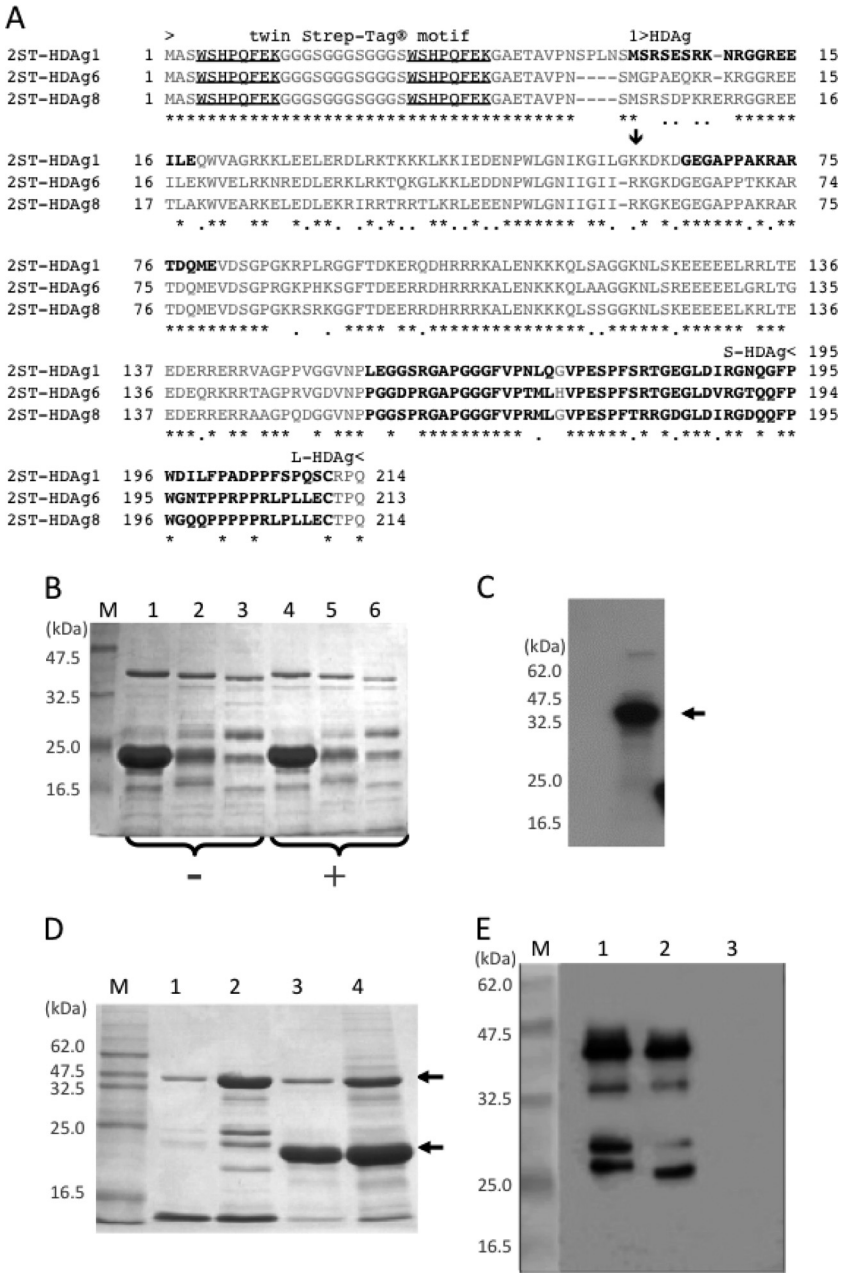

FIG 1 Twin Strep-tag–S-HDAg recombinant proteins from HDV1, HDV6, and HDV8. (A) Alignment of Strep-tag–S-HDAg amino acids from genotypes 1, 6, and 8. The amino acid numbering corresponds to the HDAg proteins. The twin Strep-tag motifs are underlined; the asterisks refer to identical amino acids, and the dots refer to a semiconserved substitution. In boldface type are the peptide sequences chosen for the array-SPRi detection (see Table S3 in the supplemental material). The arrow corresponds to the cleavage of the N-terminal domain of S-HDAg1. (B) Coomassie blue coloration of PAGE for recombinant His_6-Sumo-S-HDAg from HDV genotypes 1 (lanes 1 and 4), 6 (lanes 2 and 5), and 8 (lanes 3 and 6) in the absence (−) or presence (+) of protease cocktail inhibitors plus phenylmethylsulfonyl fluoride (PMSF). M, molecular size marker. (C) *In vitro* transcription-translation of Strep-tag–S-HDAg1 in rabbit reticulocyte lysate in the presence of [^{35}S]methionine was performed only for genotype 1. The arrow indicates the translation product. (D) Comparison of 2 extraction buffers, SoluLyse (Amsbio) (lanes 1 and 3) and BugBuster (Novagen) (lanes 2 and 4), for Strep-tag–Sumo-S-HDAg1 (lanes 1 and 2) and His_6-Sumo-S-HDAg1 (lanes 3 and 4), indicating no inhibition of protease activity for the His_6-Sumo-S-HDAg1 construction, whatever the buffer used. The upper arrow indicates the full-length HD recombinant protein, and the lower arrow indicates the NH2-terminal domain. M, molecular size marker. (E) Western blot result using anti-HDV polyclonal human antibodies. Lane 1, Strep-tag–Sumo-S-HDAg from HDV1; lane 2, His_6-Sumo S-HDAg from HDV1; lane 3, Sumo; M, molecular size marker.

GenOptics-Horiba Scientific (Chilly-Mazarin, France). Electrodeposition was performed using an Omnigrid Micro robotic arrayer (GenOptics-Horiba Scientific). The surface plasmon resonance (SPR) signals were monitored at 810 nm, using a surface plasmon resonance imager, SPRi-Plex, from GenOptics-Horiba Scientific.

Surface plasmon resonance imaging analyses. Proteins (10 μM) or peptides (100 μM) were grafted in triplicate on the gold surface of the biochip by electrochemical copolymerization, as described in Grosjean et al. (20) and Cherif et al. (22), respectively. The binding of specific Ab to probes (proteins or peptides) was monitored using surface plasmon res-

onance imaging (SPRi). In brief, Ab interactions with proteins (or peptides) induce a modification in the refractive index near the gold surface, resulting in a change of the reflectivity (ΔR) that correlates with the amount of bound Ab (23, 24). Successive serum injections (diluted 1:50 in the running buffer) were performed as described in Brakha et al. (23). To preserve the probe structure, 0.1% glycerol was added in each solution. Indeed, because the protein probes are structure sensitive during a regeneration step with 0.1 mol/liter HCl-glycine (pH 2.3), this step was immediately followed by a 10-min injection of Tris buffer (0.1 mol/liter Tris, 20 mmol/liter EDTA, 0.1% glycerol [pH 7.2]) to ensure rapid neutralization before stabilizing the chip in the running buffer (137 mmol/liter phosphate-buffered saline [PBS], 3 mmol/liter NaCl; 1.5 mmol/liter KCl; 8 mmol/liter KH_2PO_4, Na_2HPO_4 [pH 7.2]). Furthermore, for the protein probes, we also discarded a cleaning SDS step that was performed during the peptide probe processing. Twelve blank injection/regeneration cycles were performed before sample analysis to stabilize the peptide chip. This task was omitted in the case of the protein chip to reduce the number of regeneration steps that were found to affect protein integrity.

RESULTS

S-HDAg type 1 protein bears a double lysine motif that can be targeted by bacterial proteolysis. The *SHD* genes from HDV genotypes 1, 6, and 8 (Fig. 1A) were amplified and cloned into a superexpression vector, fused to Sumo protein containing a His_6 tag or a twin Strep-tag motif located at its N terminus. After protein expression in *E. coli* BL21(DE3), using autoinduction medium for 20 h at 20°C, recombinant S-HDAg proteins were extracted and purified using affinity chromatography. Purity was estimated by Coomassie blue-colored SDS-polyacrylamide gel electrophoresis and specificity by Western blot analysis using anti-HDV human antibodies. The three 35-kDa recombinant proteins His_6-Sumo–S-HDAg-1, -6, and -8 (Fig. 1) were produced and purified under these conditions. Interestingly, in the case of HDAg-1, a highly expressed 20- to 25-kDa form (Fig. 1B, lanes 1 and 4) was also observed; this form was clearly less detectable in the HDV6 and HDV8 gel lanes (see Fig. 1B, lanes 2 and 5 and lanes 3 and 6, respectively). It was possible that the expression of this truncated form was the result of proteolysis directed against a specific sequence in S-HDAg1 that occurs more efficiently than for S-HDAg from HDV genotypes 6 and 8. To test the hypothesis of a specific cleavage site, mass spectrometry analysis was performed on the purified spot (electrospray ionization [ESI]) that gave a mass of 20,428.4 ± 1.7 Da. This mass corresponded to the sequence amino acids (aa) 2 to 179 of a recombinant protein, His_6-Sumo–SHD1, which contains only the N-terminal domain S_2-K_{61} of S-HDAg1. Indeed, K_{61} was the second lysine residue of a $GKK_{61}D$ motif that was absent from both the S-HDAg6 and S-HDAg8 sequences, in which an IKRG motif was evidenced (see arrow in Fig. 1A). After Sumo protease digestion, the reactivity of full-length SHD1 and the truncated N-terminal domain was validated by HDAg detection using an ELISA (Adaltis) (data not shown). During the optimization of the production of full-length S-HDAg1, it was found that changing the His_6 tag to Strep-tag stabilized the protein conformation (Fig. 1D).

Reproducibility of SPRi experiments. The technique used in this study to analyze the humoral responses of HDV-infected patients associates protein or peptide chips with SPRi detection, which allows the screening of large numbers of samples for numerous markers, including, for example, hepatitis B and/or hepatitis C serological analyses (20, 23). However, the relevance of such an approach depends on the reproducibility of the measurements, and this point was first assessed for both the protein and peptide chips. In each case, probes were grafted in triplicate on two chips, and the serum samples were injected in random order. The presence of spots with control protein (S-HDAg1) or HCV peptide (C131) and periodic injections of a control sample (pool of serum samples from HDV-infected patients or rabbit anti-C131 serum, respectively) allowed us to monitor the reduction of signal along the successive injections, as previously described (24), and to model it using a polynomial curve (see Fig. S1 in the supplemental material). This curve was used to determine a correction factor to be applied to the SPR values obtained for the other injections.

For the protein chips, a series of injections using 15 serum samples remaining from HDV-infected patients were performed on two different chips. Indeed, not enough sera from patients 12 and 16 were available after the peptide array assay to perform multiple injections (no injection for P12, a single one for P16). The control sample was injected 5 times during the run to evaluate the repeatability of the chip efficiency during the full procedure time. The SPR signal obtained on HEL spots was used as background and subtracted from the other values. The reproducibility of the experiments was assessed by analyzing the signals obtained on the two chips for each serum on S-HDAg1 spotted in triplicate (Fig. 2A).

For the peptide chips, two series of injections using 17 serum samples from infected patients were performed on the first peptide chip and one on the second chip. The control sample was injected 8 times (first chip) and 4 times (second chip) to explore a possible loss of efficiency of the chip during the experiment. The SPR signal obtained on the HEL spots was used as background noise and subtracted from the other values. The reproducibility of the experiment was assessed by analyzing the sum of the signals obtained on all peptide spots for each serum (Fig. 2B). In both cases (protein and peptide chips), the reproducibility was good, and the coefficient of variation did not exceed 20% for low Ab concentration.

Humoral response against S-HDAg1 protein and N-terminal domain. Four proteins were grafted on a protein chip: S-HDAg1, the N-terminal domain of S-HDAg1, Sumo, and HEL. Sixteen serum samples from infected patients, as the serum from P12 (patient 12) was not available after the peptide array assay (see below), and 3 healthy donor samples were injected as described, and the SPR signals were analyzed after subtraction of the background noise (signal on HEL spots) and correction for the decrease of the signal during sample screening. As shown in Fig. 3, Abs against S-HDAg1 were detected in the majority of the infected patients (13/16 [81.2%]), whatever the genotype of the infecting virus. The amplitude of the SPR signal (ΔR), corresponding to the amount of bound Ab on the probe (i.e., the amount of Ab present in the serum) (24), varies from serum to serum but seemed to be independent of the infecting genotype. The specificity of the SPRi signal was supported by the absence of significant ΔR values on the control protein Sumo. The presence of Ab specific for the N-terminal S-HDAg1 domain was found to be less constant; however, the ΔR values collected on N-terminal protein spots were significant compared to the control spots grafted with Sumo ($P < 0.1\%$) (Fig. 3).

Humoral response against HDV peptides. Fourteen different peptides were grafted on a chip (see Table S2 in the supplemental material). Eleven of them corresponded to HDV S-HDAg- and

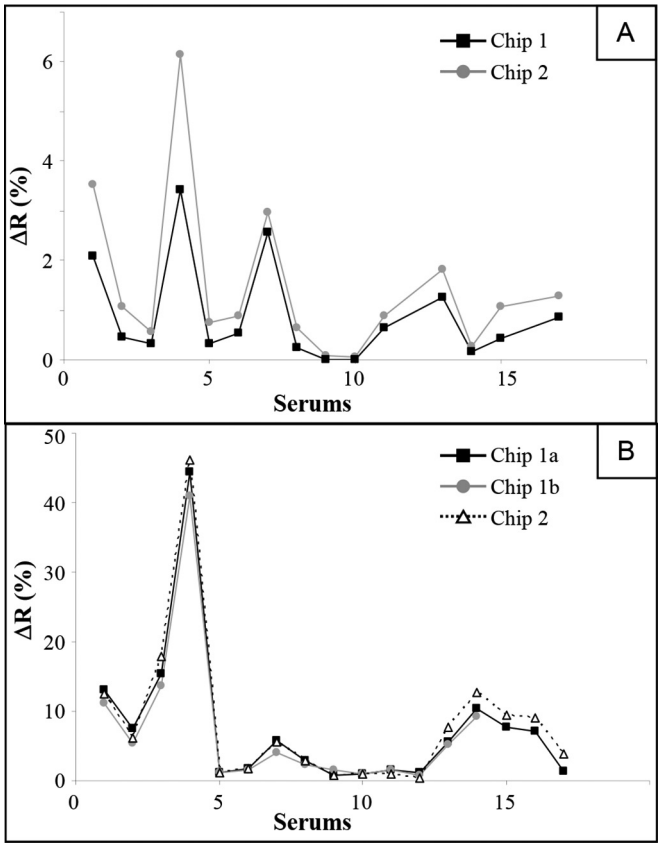

FIG 2 Reproducibility of the SPRi experiments. Two chips were grafted with probes in triplicate, and serum samples from infected patients were successively injected in random order. A regeneration step took place between each injection, and both background noise (control spots) and the reduction of signal along the experiment (control sample) were taken in account. (A) Protein chips were grafted in triplicate with S-HDAg1 (control spot, HEL; control sample, pool of HDV-positive sera). The SPR signals obtained on the S-HDAg1 spots for each serum were analyzed. (B) Peptide chips were grafted in triplicate with 11 HDV peptides (control spots, HEL101 and C131; control sample, rabbit anti-C131 immune serum). Two series of injections were realized on the first chip and one on the second. The sum of the SPR signals obtained on all HDV peptide spots for each serum was analyzed. The bars represent the standard deviation (SD).

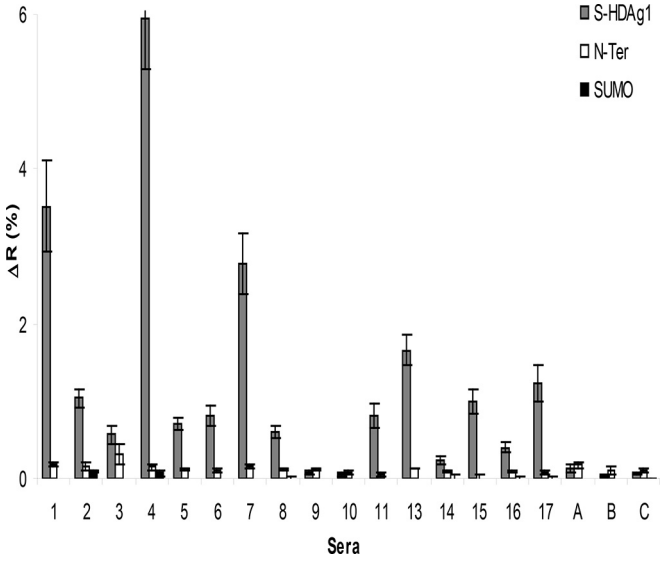

FIG 3 Humoral response against S-HDAg1. S-HDAg1 and N-terminal (N-Ter) fragments were immobilized in triplicate on a chip surface, and serum samples from 16 infected patients (patients 1 to 11 and 13 to 17, as no more serum was available from patient 12) or from healthy donors (A to C) were successively injected. The spots grafted with Sumo were used as controls. The SPRi signals were analyzed for each serum. The bars represent the standard deviation (SD).

L-HDAg-specific sequences: five from HDV genotype 1, three from HDV genotype 6, and three from HDV genotype 8. We also grafted three control peptides, HEL101, Ova75, and C131, the last one being used to determine the loss of efficiency of the chip (see "Reproducibility of SPRi experiments" above). The sera were injected as described, and the SPRi signals were analyzed after subtraction of the background noise (signal on HEL101 spots) and correction for the decrease of the signal during sample screening. The results are summarized in Table 1. For detailed data, see Fig. S3 to S6 in the supplemental material. As observed for Abs against the whole HDV protein, a majority of the tested patients (15/17 [88.2%]) had developed Abs specific for HDV peptides, but the specificity and the amount of these Abs varied among each case. However, as nine patient samples led to a significant SPR signal on peptide 65 type 1 (65#1), it appears that epitope 65-80 might be immunodominant, whatever the infecting genotype. In contrast, only one serum sample presented >0.5% reactivity on peptide 1#1. Moreover, we observed serological cross-reactivities between the different genotypes, despite the lack of complete identity between the target peptides (see Table S3).

Nature of anti-HDV Abs present in patient serum. To determine if anti-HDV Abs present in the sera corresponded to the IgG and/or IgM, two serum samples from high responders (P4 and P7) were first injected on a chip grafted with S-HDAg1. After the washing step and before the regeneration step, either anti-human IgM or anti-human IgG was injected. A high SPR signal was observed after anti-IgG injection, whereas no signal was detected after anti-IgM injection. Thus, only anti-IgG bound on the Ab linked to the grafted HDV protein was detected using SPRi, despite the fact that in the remaining available serum from P4, both IgM anti-HDV and IgG anti-HDV were detected using the DiaSorin commercial assay (data not shown).

DISCUSSION

By determining the amino acid residues exposed on the HDV ribonucleoprotein (RNP) using immunoprecipitation and Pepscan analysis, Bichko et al. (25) showed that among 17 *in vitro*-generated antibodies screened, three HD regions (nuclear localization signal [NLS], the carboxy-terminal part of S-HDAg, and the carboxy-terminal extension of L-HDAg) were recognized on the RNP by immunoprecipitation (25). Two Abs (7gp3 and 8gp4) recognize the so-called core sequence of the bipartite NLS (aa 67 to 88) (26), four out of five Abs specific for the carboxy-terminal part of S-HDAg were immunoreactive, and finally, the carboxy-terminal extension specific to L-HDAg was also recognized. Interestingly, the first domain of the NLS exposed in the HDV RNP, as shown in Bichko et al. (25), was explored using peptide 65#1, including the PPAKRAR sequence; this peptide gave the most sensitive response in the SPRi approach, as 9 serum samples out of 17 were clearly reactive (see Table 1). It is interesting to note that this region was also recognized by HDAg-specific $CD4^+$ T-cell clones

TABLE 1 Humoral response against HDV in infected patients: Ab specificity

Grafted probe genotype by patient	Reactivity (SPR signal [%]) in[a]: Protein array (S-HDAg1) (genotype 1)	Peptide array by genotype: 1# (genotype 1)	65# (genotype 1)	155# 1	155# 6	155# 8	174# 1	174# 6	174# 8	189# 1	189# 6	189# 8
Genotype 1												
P5[b]	+	−	−	−	−	−	−	−	−	−	−	−
P8	+	−	++	−	−	−	−	−	−	+	−	±
P14	−	−	++	−	−	−	+	−	±	−	−	−
P15	+	+	++	−	−	−	±	−	−	+++	±	±
Genotype 6												
P9	−	±	−	−	−	−	−	−	−	−	−	−
P10[b]	−	−	−	−	−	−	−	−	−	−	−	−
P16	±	±	++	±	−	−	−	−	−	−	+	++
P17	++	−	+	+	−	−	±	−	−	−	−	−
Genotype 8												
P2	++	±	++	−	−	−	−	−	−	−	−	−
P12	ND	−	+	−	−	−	−	−	−	−	−	+++
Genotype 5												
P1	++	−	−	+	±	+++	++	−	−	−	±	±
P4	+++	+	++++	++	−	±	++	+++	++++	+	++	+++
P6	+	−	−	−	−	−	−	−	−	−	−	±
P11	+	−	−	−	−	−	+	−	−	−	−	−
Genotype 2												
P7	++	−	++	−	−	−	±	−	−	−	−	±
Genotype 7												
P3	+	±	±	−	−	−	±	++	++	−	++	+++
P13	++	−	−	−	−	±	−	−	−	−	−	−

[a] SPR signal obtained on S-HDAg1 and different HDV peptide spots after injections of serum samples from patients. The symbols for the SPR signal (%) are −, <0.25; ±, >0.25 and <0.5; +, >0.5 and <1; ++, >1 and <5; +++, >5 and <8; ++++, >8. Sera from healthy donors led to a signal of ≤0.25%. ND, not done.
[b] Note that the serum samples from P5 and P10 gave no positive results against peptides.

from 3 HDV-infected individuals having asymptomatic HDV disease (27). Because this region was absent in the N-terminal domain of S-HDAg1, it might partly explain the overall low Ab reactivity of this protein domain (Fig. 3). Furthermore, homodimers of peptides 12 to 60 have been implicated in the formation of an antiparallel hydrophobic core that can lead to an octameric structure possibly involved in the polymerization of the HDAg (28). In addition, this region was not exposed on the viral RNP (25). Finally, the HDV1-derived NH_2 terminal peptide 1 (aa 1 to 18) also was not immunogenic among the 17 serum samples studied here. Together, this would lead one to consider that this domain is of low immunogenicity. In another study that explored linear epitopes through a systematic overlapping peptide mapping from the full-length L-HDAg type 1 sequence, Wang et al. (29) demonstrated that five serum samples having high anti-HDV titers were reactive against 7 regions. Hexamers corresponding to the aa 63 to 74 domain were recognized by serum samples from chronically infected humans and woodchucks; furthermore, peptides corresponding to the carboxy-terminal part of the S-HDAg (aa 159 to 172 and aa 174 to 194) reacted with serum antibodies from all five human serum samples studied (29). Here, to explore some Ab responses to S-HDAg carboxy-terminal regions, we compared the reactivities of 17 human serum samples against type 1-, 6-, and 8-specific peptides in two regions, those of 155 and 174 (see Table S2 in the supplemental material). Overall, the results we obtained indicated that using peptide array platform and SPRi detection, such S-HDAg carboxy-terminal regions were found to be reactive for 10 HDV-infected individual serum samples among the 17 studied patients, regardless of the infecting genotype (Table 1). For example, serum from patient 1 (P1), who was infected by HDV5, was strongly reactive against peptides 155#8 and 174#1 but remained negative against 174#6 and 174#8. A serum sample from P3, who was infected with HDV7, remained negative against peptide 155 toward all different genotype sequences but had a strong reactivity against peptides 174#6 and 174#8. In all, these results indicate that expanding the diversity of the target sequences enhanced the sensitivity of this array approach.

During the experimentation, some limitations of the approach were noticed. Indeed, using SPRi, it was observed that (i) the use of full-length delta protein was fairly delicate, due to the denaturation of the protein during the washing step, (ii) optimization for diagnostic purposes should take into account the improvements in automation, (iii) it was not possible to test anti-HDV antibody reactivities during acute HBV-HDV coinfection samples, as all samples corresponded to chronically infected patients, (iv) no samples corresponded to patients infected with HDV3 or HDV4,

and (v) no specific anti-S-HDAg IgM reactivity was found, although the two samples chosen to be tested had high anti-HDV titers. This might be due to the low affinities of IgM Abs under our conditions.

Considering the region encompassed by peptides 155 and 174, it has been shown in previous work exploring the *HD* gene evolution in chronically infected patients that most positively selected sites from 5 patients studied were located within this B-cell immunogenic domain (30). Interestingly, we also previously characterized S-HDAg sequences in HDV-infected patients on a high-dose interferon treatment regimen and found that the 169 to 172 aa domain was hypervariable and might represent an important selected epitope after 1 year of interferon therapy, when the replication recurred (P. Dény, unpublished data). The discrepancy observed between the patient Ab response against these 2 regions (Table 1) might also be associated with the level of expression of each HDAg isoform: in a mouse model, using vaccination with plasmids expressing S-HDAg, L-HDAg, or a nonfarnesylated form of L-HDAg-C_{211}S mutant, it was suggested that the L-HDAg vaccination response hardly reacted with epitope aa 174 to 194, in contrast to the S-HDAg or L-HDAg C_{211}S mutant vaccination responses (31).

One series of the designed type-specific peptides (189#1, #6, and #8) encompasses the junction between the very carboxy-terminal end of S-HDAg extending through tryptophan 196 (W_{196}) to the cysteine (C_{211}) involved in the farnesylation signal C_{211}XXQ present in all L-HDAg sequences from HDV strains, regardless of the genotype. Interestingly, the carboxy-terminal part of this peptide sequence is quite different between HDV genotype 1 (P_{204}PFSPQSC_{211}) and African non-1 type sequences (P_{204}RLPLLEC$_{211}$) (12). In addition, it is of interest to note that a specific aa 202 polymorphism (S202A) differentiates African from European L-HDAg HDV type 1 (32). It is therefore not surprising to observe that when a specific reaction occurred against this peptide, the specificity was genotype dependent. Indeed, for these regions, serum samples from patients infected with genotypes 5, 6, 7, and 8 reacted more strongly against type 6 and 8 peptides (from African HDV-derived sequences) than against type 1 peptides; conversely, serum samples from patients infected with type 1 (P8 and P15) reacted fairly against type 1-specific peptides.

In conclusion, we have developed a promising protein-peptide array to assess the anti-HDV antibody response in order to explore the etiology of any cytolytic hepatitis syndrome. Using well-designed different protein-peptide sequences can lead to efficient screening that may characterize viral (and/or other infectious agent) infections. This approach would also certainly be helpful in reducing the underestimation of such HDV satellite infections that unfortunately remain neglected due to the lack of efficient therapies. It would therefore contribute to the specification of the importance of HDV in countries where information about HDV prevalence among HBsAg-positive patients is lacking. This work provides evidence confirming that the amino-terminal part of the HD protein is not useful for searching for HDV-specific antibody responses. In contrast, epitopes exposed on the RNP, as previously described (25), seem to be good candidate targets. SPRi detection is more adapted to peptide probes than to proteins, the structure of which resists the denaturing process less efficiently. Finally, analyzing different regions of HD proteins is mandatory for improving the sensitivity of such tests, because for some peptides, the antiviral Ab response seemed to be genotype specific.

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